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INSECTICIDAL POTENTIAL OF *Abelmoschus moschatus* TRYPSIN INHIBITOR (AMTI-II) AGAINST MID GUT PROTEASE OF *Helicoverpa armigera*

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ABSTRACT

The present study was aimed to test the efficacy of Abelmoschus moschatus trypsin inhibitor (AMTI-II) against the isolated mid gut protease of Helicoverpa armigera. A potent trypsin inhibitor, AMTI-II, has been purified and characterized from the seeds of Abelmoschus moschatus following conventional methods of protein purification and its molecular weight was found to be 21.2 kDa by SDS-PAGE. The inhibitor was quite stable up to 80°C for 10 min and was not affected at alkaline as well as acidic conditions tested. The mid gut protease was found to be homogeneous with a molecular weight of about 30.4 kDa. The isolated protease was alkali stable and its pH optimum for activity was about pH 11. The isolated protease was found to be susceptible to inhibition by AMTI-II with an IC_{50} value of 2.2 µg. The mode of action of AMTI-II on the mid gut protease was found to be non-competitive. Results of kinetic studies revealed a low Ki value of 8×10^{-8} M against the protease. On molar and quantitative basis, AMTI-II appears to be the most powerful inhibitor than SBTI. AMTI-II exerted a strong inhibition on the isolated mid gut protease of Helicoverpa armigera, a property that helps in exploring its use in the generation of transgenic for combating the insect pest.

KEYWORDS: Protease İnhibitors, Helicoverpa Armigera, SDS-PAGE, Non-Competitive İnhibition, Soybean Trypsin İnhibitor (SBTI), Transgenics

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Original Article

INTRODUCTION

Plants produce a group of secondary metabolites, also called allelo chemicals - lectins, tannins, alkaloids, polyphenols, saponins, phytates, antibiotics, cyanogenic glycosides, enzymes, flavonoids, enzyme inhibitors etc. in response to attack by insect pest and pathogens (De Leo *et al.*, 2001) and interfere with the digestion process of insect, affecting the metabolism, disrupting growth and development of the larva (Jadhav *et al.*, 2012a). Among them, protease inhibitors regulate physiological processes through controlling protease activities and are also involved in defense mechanisms in plants (Kim *et al*., 2006; Lopes *et al.*, 2009). These proteins interact with their target proteases and form stable protease-inhibitor complexes that are incapable of enzymatic activity (Norton, 1991). These protease inhibitors have also been shown to act as defensive compounds against phytophagous insects by the direct assay or expression in transgenic crop plants (Koiwa *et al.*, 1998, Vain *et al.*, 1998). These proteins may act either alone or in concert to contribute to overall resistance to plants and occur as constitutive parts of plant tissues and are often inducible.

Protease inhibitors are known to disrupt protein digestion in insects by inhibiting mid gut proteases, impede the supply of essential amino acids for the formation of proteins (Broadway and Duffey, 1986; Delano *et al.*, 2008) and therefore results in the reduction of growth and development of insect by interrupting protein digestion (Naseri *et*

al., 2010, Shaikh *et al.,* 2014). This anti-nutritional effect is explored through developing transgenic containing plant genes encoding protease inhibitors for withstanding insect attack (Dunse *et al.,* 2010). Insects, however, tend to adapt to this situation by over producing protease inhibitor sensitive proteases and / or up regulating the expression of proteases that are insensitive to the inhibitors or inducing production of protease inhibitor degrading enzymes (Bolter *et al.,* 1995; Zhu-Salzman *et al.,* 1998; Cloutier *et al.,* 2000).

Exogenous application of pesticides to combat insect attack has become less feasible, mainly due to the development of pesticide resistance in insects and inherent possible environmental hazards (Armes *et al.,* 1996). The application of genetic engineering for the transformation of crop plants for insect resistance has revolutionized conventional breeding. The *Bacillus thuringiensis* (BT) endotoxin gene has been successfully expressed in several crops to impart resistance against herbivorous insects (Jouanin *et al.,* 1998). However, insects have developed resistance to Bt endotoxin by producing a proteinases that inactivates the toxin (Oppert *et al.,* 1996; Michaud, 1997) or by lacking the proteinase allele required for activation of Bt protoxin (Oppert *et al.,* 1997). With increasing resistance developed by insects to a wide range of pesticides, there is a need to develop crop cultivars that are resistant to insects.

Numerous insect-feeding bioassays and experiments with transgenic plants have also shown the delayed growth and development of the insect (Koiwa *et al.,* 1998, Parde *et al.,* 2010). When ingested by an insect, they result in starving of the insect for amino acids by inhibiting digestive proteases and thus retarding the growth and development (Giri *et al.,* 2005). Transforming plant genomes with these proteinaceous protease inhibitors provide safe approach to pest control (Reckel *et al.,* 1997). However, because of the variability of the insect proteases and the restricted range of action of the protease inhibitors (Ortego *et al.,* 1996), the expression of a particular protease inhibitor in plants may not yield the desired result and therefore might not be an excellent candidate for biotechnology. Thus there is a continuous search for new inhibitors that are competent to combat pest adaptation.

Helicoverpa armigera (Lepidoptera: Noctuidae) is a polyphagous pest of 182 plant species across 47 families in the Indian subcontinent, out of which 56 are heavily damaged and 126 are rarely affected. *Helicoverpa armigera*, also known as cotton bollworm or legume pod borer, is one of the most devastating crop pest of cotton, pigeon pea, chickpea, groundnut, sorghum, pearl millet, tomato and others of economic importance (Bhavani *et al.,* 2007; Wondafrash *et al.,* 2012). It decreases crop yield by chewing leaves, flowers, green pods and developing seeds. Because of its high mobility, survival rate under adverse conditions, capacity to complete several generations in a year and ability to develop resistance against insecticides, its management is very difficult. Due to indiscriminate use of insecticides to control this pest, it has developed a high level of resistance to conventional insecticides (Kranthi *et al.,* 2002).

The larval mid gut harbors proteases which hydrolyze the proteins to amino acids essential for their growth and development. Of the four classes of proteolytic enzymes, serine proteases are among the best studied proteases from the insect mid gut (Hau and Benjakul 2006, Mohammadi *et al.,* 2010). Larval mid gut contains a complex enzymatic system which helps in protein digestion and studies on the digestive proteases of lepidopteran insects have revealed that 95% of total digestive activity relies on serine proteases (Ghodke *et al.,* 2013). Serine proteases, trypsin and chymotrypsin predominate digestive tract of *Helicoverpa armigera*. Studies have shown that a major part of *Helicoverpa armigera* gut protease activity can be blocked by soybean kunitz trypsin inhibitor (Johnston *et al.,* 1991, Harsulkar *et al.,* 1999). Health and environment hazards of synthetic pesticides along with developed resistance of insect towards chemical pesticides have made researchers to explore other ways to confer insect resistance (Kranthi *et al.,* 2002). Since there is significant

variation among the biochemical properties of insect digestive proteases, their isolation and characterization is necessary for designing a safe control strategy that utilizes plant-proteinaceous inhibitors (Wilhite *et al.,* 2000). Therefore, it is important to develop alternative methods of controlling this pest, including host plant resistance.

Reports supporting protease inhibitors in retarding growth and development of *Helicoverpa armigera* on their incorporated diets as well as on transgenic plants expressing their genes are also available (Harsulkar *et al.,* 1999; Telang *et al.,* 2003; Srinivasan *et al.,* 2005). A significant reduction in larval weight and increase in mortality rate were observed when protease inhibitors from *Capsicum annuum*, chickpea and kidney bean were incorporated in artificial diet for bioassay during feeding trials of *H. armigera* (Tamhane *et al.,* 2005; Kansal *et al.,* 2008; Mittal *et al.,* 2014**).** It has also been reported that crude/purified protease inhibitor from different sources inhibits digestive enzymes such as trypsin and chymotrypsin which results in larval and pupal growth reduction (Naseri *et al.,* 2010, Pandey *et al.,* 2014).

Knowledge of enzyme properties together with their sensitivity to various inhibitors can provide a base for the control methods. Feeding studies using isolated inhibitor supplemented in the diet of insects are laborious and time consuming. The effects of protease inhibitors on insect digestive enzymes can be measured directly by means of enzyme assays carried out *in vitro* (Christeller *et al.,* 1992). Studying the effect of inhibitor on pure protease isolated from mid guts could provide a better picture about the potency of the inhibitor as an insecticidal agent. Thus, the present investigation was focused on the purification of trypsin like protease from the mid gut of *Helicoverpa armigera* larvae and the potent inhibitory effect of trypsin inhibitor, AMTI-II on the isolated mid gut protease of *Helicoverpa armigera.*

MATERIALS AND METHODS

Chemicals and Reagents

Bovine pancreatic trypsin (1× crystallized, DCC-treated, type xi), α-N-benzoyl-DL-arginine-p-nitoanilide HCl (BAPNA), Phosphorylase b, Bovine serum albumin (BSA), Ovalbumin, Carbonic anhydrase, SBTI (soybean trypsin inhibitor), Blue dextran, Acrylamide, N,N'-methylene bis acrylamide, Sodium dodecyl sulfate (SDS), N,N,N',N'-Tetramethylethylenediamine (TEMED), Coomassie brilliant blue R250 were purchased from Sigma Chemical company, St. Louis, Missouri, U. S. A. Sephadex G-200 and QAE-Sephadex were purchased from Pharmacia Fine Chemicals, Upsala, Sweden. All other chemicals used were of analytical grade.

Purification of *Abelmoschus moschatus* Trypsin Inhibitor (AMTI-II)

Abelmoschus moschatus trypsin inhibitor (AMTI-II) has been previously isolated and purified following ammonium sulphate fractionation, DEAE-cellulose ion exchange chromatography and gel permeation on Sephadex G-100 (Muni Kumar *et al.,* 2015).

Larval Mid Gut Enzymes

Helicovera armigera larvae grown on chick pea fields were collected from Central Tobacco Research Institute (CTRI), Rajahmundry. In order to avoid any fluctuations in activities due to physiological and environmental conditions, larvae were synchronized before assaying for the gut proteases. This was accomplished by selectively separating the fifth instar larvae and allowing them to feed on chick pea pods *ad libitum* for 24 h. The specimens killed by drowning in distilled water were immediately dissected over ice. The mid guts were transferred on to a watch glass containing ice cold 0.1% NaCl and gently cleaned with a brush to remove undigested food particles or adhering fat bodies. Mid gut tissue was

then stored at -20^0 C until further use.

Purification of Trypsin like Protease from *Helicoverpa armigera*

The purification procedure has been carried out with several batches of larvae. Nearly identical protein profiles were obtained each time but with some variation (10-15% in enzyme activity). Serine proteinase from mid gut extracts of 5^{th} instar larvae was isolated and purified following conventional methods of protein purification.

The mid guts obtained from fifth instar larvae were homogenized with one ml of ice cold extraction buffer, 10mM Phosphate buffer, pH 7.6 and centrifuged at 10,000 rpm at 4^o C for 10 min. To the supernatant, four volumes of ice-cold chloroform was added and mixed thoroughly before subjecting it to centrifugation at 3,000 rpm at 4^o C for 10 min. The aqueous fraction was then carefully pipetted out and collected. The pigments that occurred as a part of larval food and contaminating pigments from the larval cuticle were removed in this step.

To the aqueous fraction, ammonium sulphate was added to obtain 60% saturation at 4^oC with constant stirring. The precipitated proteins were centrifuged at 10,000 rpm at 4^o C for 30 min and the pellet was then dissolved in a minimum volume (one ml) of 20mM Tris-HCl buffer, pH 7.2. Loaded on to a QAE-Sephadex column (0.5 x 30cm) equilibrated with the same buffer. The column held at < 8^o C was washed with 20mM Tris-HCl buffer, pH 7.2 and then eluted with 0.1 - 0.3M NaCl prepared in the same buffer. Fractions, each 2ml, were collected at a flow rate of 20ml/h and the protein content was monitored at 280 nm. The enzyme activity in each fraction was determined using BAPNA as the substrate.

Estimation of Trypsin Activity

Amidolytic activity of trypsin was assayed by the method of Kakade et al. (1969) using BAPNA as the substrate. Substrate solution was prepared by dissolving 30 mg of BAPNA in 2 ml of dimethyl sulfoxide and the solution was made up to 100 ml with 50 mM Tris-HCl buffer, pH 8.2 containing 20 mM $CaCl_2$. Aliquots of trypsin solution containing 10-50 µg of the enzyme in 2 ml distilled water were incubated with 7 ml of BAPNA solution at 37°C for 10 min. The reaction was stopped by adding 1 ml of 30% acetic acid. The absorbance of the samples was then measured at 410 nm against a blank.

For trypsin inhibitory activity, purified trypsin (10-50µg) like protease was pre-incubated at 37 °C for 10 min with aliquots of the inhibitor and the residual trypsin activity was taken as an index of the inhibitory activity. Suitable controls were included to correct for the presence of endogenous proteinase activity in the extract. One trypsin unit is defined as an increase in 0.01 absorbance unit at 410 nm for trypsin under the assay conditions. One trypsin inhibitory unit is defined as the number of trypsin units inhibited under assay conditions.

Protein Estimation

Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard. The protein content in the column effluents collected during chromatographic separation was determined by measuring the absorbance at 280 nm.

Molecular Weight by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out by the method of Laemmli (1970) in slab gels. The marker proteins, phosphorylase b (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), Carbonic anhydrase (29kDa), soybean trypsin inhibitor,

SBTI (20.1 kDa) and lysozyme (14 kDa) in 0.1 ml of phosphate buffer were kept at 100°C for 2 min with 0.1 ml of sample buffer (0.5 M Tris-HCl buffer, pH 6.8 containing 4% SDS and 10% 2-mercaptoethanol). The protease in sample buffer was kept at 100°C for 2 min and then subjected to electrophoresis. After electrophoretic run, proteins were fixed in glacial acetic acid 10% (v/v), methanol 30% (v/v) and were visualized using coomassie brilliant blue.

Effect pH and Temperature on Mid Gut Protease

In order to determine the pH stability of protease, enzyme in an appropriate buffer (pH range of 3-12) was kept at 5^0C for 24 h, aliquots were then taken and assayed for enzyme activity using BAPNA as the substrate as described earlier. Buffers used were Citric acid-Sodium citrate, 0.1 M (pH 3-4), Acetic acid-Sodium acetate, 0.1M (pH 5-6), 0.1 M Sodium phosphate buffer (pH 7-8), Tris- HCl, 0.1M (pH 9), 0.1M glycine-NaOH (pH 10) and Sodium bicarbonate-sodium carbonate buffer (pH 11-12).

In order to determine the temperature stability of protease, enzyme (2ml in 0.1M sodium phosphate buffer, pH 7.6) was incubated for 30min at different temperatures (20-80^{o}C). After cooling for 2h, enzyme activities were determined from the aliquots using BAPNA as the substrate.

Effect of Various Metal Ions and Protease Inhibitors

Metal ions (Mg^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+}, Cd^{2+}, Mn^{2+}, Hg^{2+}) were tested for their inhibitory effect. Reaction mixture containing 50 µl enzyme (25µg), 5 mM metal ion and 150 µl assay buffer (0.1M sodium phosphate buffer, pH 7.6) was incubated for 20 min at 37^{o}C. Reaction was started by adding 800 µl BAPNA (30mg/100ml) and trypsin activity was measured.

The effects of various protease inhibitors such as TLCK, TPCK, Iodoacetate and EDTA each 5mM on proteolytic activity of isolated enzyme were investigated. After 30 min of pre-incubation of inhibitors with enzyme at room temperature, substrate was added and residual protease activity was measured by the standard assay method.

Mode of Inhibition of AMTI-II on Mid Gut Protease

The amidolytic protease activity of mid gut protease of *Helicoverpa armigera* in the absence and presence of AMTI-II (1 µg, 2 µg and 3 µg) and Soybean trypsin inhibitor, the established trypsin inhibitor with insecticidal activity (1 µg, 2 µg and 3 µg) (SBTI) was measured at different concentrations (0.9-4.0 mM) of BAPNA. Dixon plot analysis was employed to determine the constants of inhibition for mid gut protease by pre-incubating the enzyme with increasing concentrations of AMTI-II and SBTI. Kinetic analysis of AMTI-II was carried out following the standard protocol and the Ki value of the AMTI-II was calculated from Dixon plot (Dixon, 1953).

RESULTS

Purification and Characterization of AMTI-II

AMTI-II was purified to apparent homogeneity following ammonium sulphate fractionation, ion-exchange chromatography on DEAE-cellulose and gel permeation on Sephadex G-100. The molecular weight of AMTI-II as determined by SDS-PAGE was 21.2 kDa (Figure 1). AMTI-II exerted strong inhibition towards bovine pancreatic trypsin and it showed moderate inhibition towards elastase. The inhibitor was quite stable up to 80°C for 10 min and was not affected at alkaline as well as acidic conditions tested. Treating with 8 M urea and 1% SDS for 24 h at room temperature did not result in any loss of antitryptic activity (Muni Kumar *et al*., 2015).

Purification of Trypsin like Protease from the Mid Guts of *Helicoverpa armigera*

A trypsin like protease has been isolated and purified from the mid guts of fifth instar larvae of *Helicoverpa armigera* following conventional methods of protein purification. When ammonium sulfate precipitated protein was subjected to Ion-exchange chromatography on QAE-Sephadex, a symmetrical peak with protease activity was eluted with 0.3M NaCl in 20mM Tris-HCl buffer, pH 7.2. The protein profile on ion exchange chromatography is shown in Figure 2. Recoveries and relative purification at each step for a typical purification from five larvae are shown in Table 1. The specific activity of the purified enzyme was 1426.92 TU per mg protein. The final yield was 52.81% of the original protease with a fold purification of 3.81. The molecular weight of isolated mid gut protease as determined by SDS-PAGE was found to be 30.4 kDa (Figure 3).

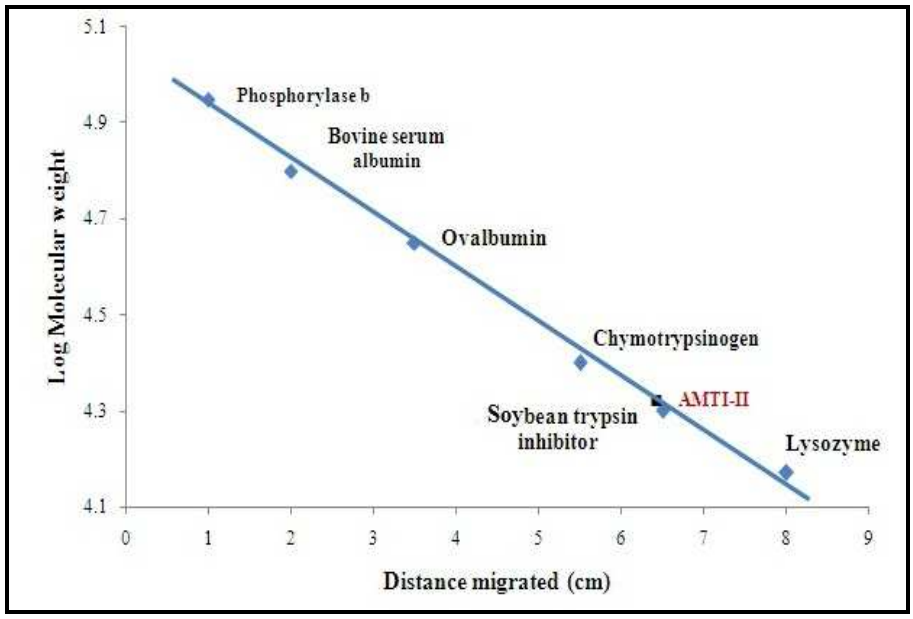


Figure 1: Molecular Weight Determination of AMTI-II by SDS-PAGE

Plot of the distance migrated in cm Vs log molecular weight of standard proteins (♦) and AMTI-II (.)

Standard Proteins

- Phosphorylase b, 97kDa
- Bovine serum albumin, 67kDa
- Ovalbumin, 45kDa
- Chymotrypsinogen A, 25kDa
- Soybean trypsin inhibitor, 20.1 kDa
- Lysozyme, 14kDa

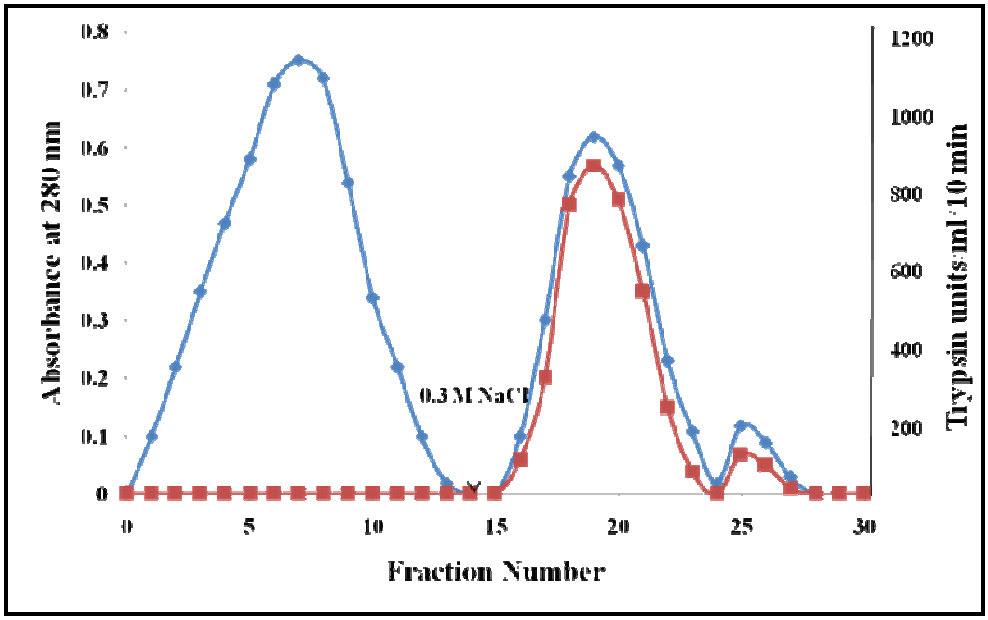


Figure 2: Ion exchange Chromatography on QAE-Sephadex of the Ammonium Sulphate Fraction

About 4.5 mg of the ammonium sulfate precipitated protein was loaded on to a column (0.5 x 30 cm) in 20mM Tris-HCl buffer, pH 7.2. Elution was done with 0.3 M NaCl in the same buffer. Two ml fractions were collected at a flow rate of 20 ml/h. Protein was monitored by measuring absorbance at 280nm (- ♦ -) and the trypsin- like activity (- ■ -) was assayed using BAPNA as substrate.

Table 1: Purification Table of Trypsin-like Protease from Mid Guts of *Helicoverpa armigera*

Preparation	Total Volume(ml)	Total protein(mg)	Total Activity(TU)	Specific Activity TU/mg Protein	Yield%	Fold Purification
Crude Extract	1.0	7.5	2810	374.67	100	1
Ammonium sulphate precipitate	0.25	4.5	1812	402.67	64.48	1.07
QAE Sephadex fraction	1.0	1.04	1484	1426.92	52.81	3.81

One trypsin unit (TU) is arbitrarily defined as an increase in 0.01 absorbance unit at 410nm per 2.5 ml reaction mixture, under the assay conditions. Yield and fold purification were calculated from activity units and specific activity respectively.

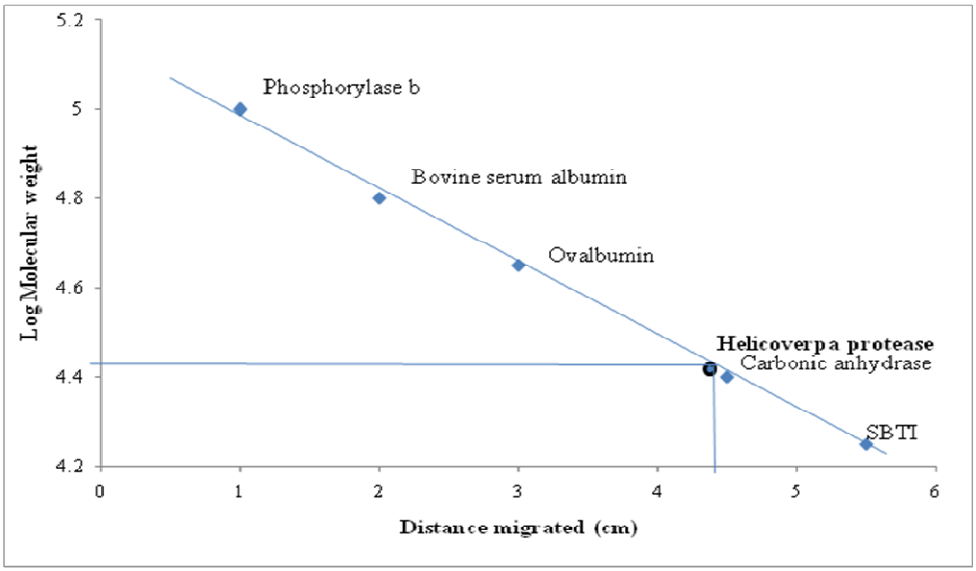


Figure 3: Molecular Weight Determination of *Helicoverpa Armigera* Protease by SDS-PAGE at pH 8.3 on 5-15% Slab Gels under Denaturing Conditions

- Standard proteins
  - Phospohorylase b (97kDa)
  - Bovine serum albumin (67 kDa)
  - Ovalbumin(45kDa)
  - Carbonic anhydrase (29kDa)
  - SBTI (20.1kDa)
- *Helicoverpa armigera* Protease

Effect of pH and Temperature on Protease Activity of Trypsin Like Protease

The enzyme was found to be unstable at acidic pH (Results not shown), whereas at alkaline pH it showed wide range of activity. The maximum enzyme activities were observed at a pH range of 9.0– 11.0 (Figure 4).

The thermal stability of trypsin-like protease in the midgut samples pre-incubated for 5 min remained unchanged at temperatures up to 40°C. However, proteolytic activity was reduced, which was almost abolished above 60°C (Figure 5).

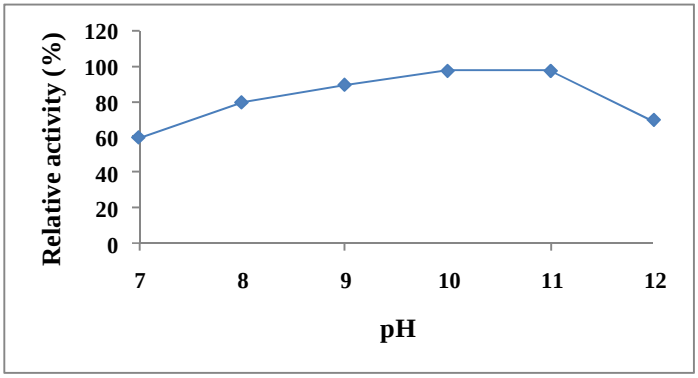


Figure 4: pH Stability of Trypsin like Protease of *Helicoverpa armigera*

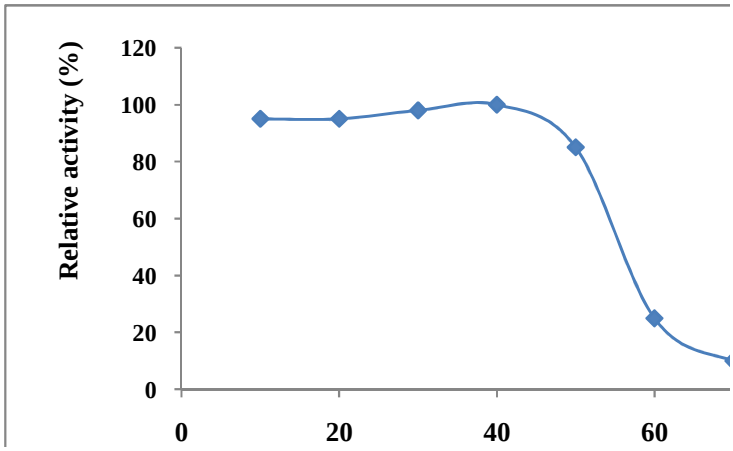


Figure 5: Thermal Stability of Trypsin like Protease of *Helicoverpa armigera*

Effect of Various Metal Ions on Protease Activity

$ZnSO_4$, $CuSO_4$, $CdCl_2$ and $HgCl_2$ at 5 mM concentration inhibited the trypsin like activity of gut protease, whereas Ca^{2+}, Mn^{2+} and Mg^{2+} at that concentration elevated the enzyme activity (Table 2). Maximum inhibition was observed with Cd^{2+} (82%) and Hg^{2+} (79%) and minimum inhibition with Cu^{2+} (45.2 %). Metal ions such as Cd^{2+} and Hg^{2+} act as strong inhibitors of enzymatic activity. Addition of $CaCl_2$, $MgCl_2$ and $MnCl_2$ at low concentrations increased the rate of trypsin activity whereas the reaction rate was progressively lowered with the increase in ionic strength (Results not shown).

Table 2: Percent Activity of Purified Trypsin like Protease after Treating with Metal Ions

Sl. No.	Metal Ion (5 mM)	Relative Activity (%)
	Control	100
1	Mg^{2+}	122.5
2	Zn^{2+}	65.3
3	Ca^{2+}	106
4	Cu^{2+}	55.2
5	Cd^{2+}	17.8
6	Mn^{2+}	115.4
7	Hg^{2+}	21.3

Effect of Inhibitors on Protease Activity

Larval midgut proteases were further characterized using protease specific inhibitors (5 mM). Calculating inhibition percentage of BAPNA hydrolysis in the presence of various inhibitors offers information about the relative contribution of the inhibited class of protease to isolated gut protease activity. From Figure 6, it was clear that protease activity was inhibited by TLCK but not TPCK indicating trypsin like but not chymotrypsin like specificity of serine protease.

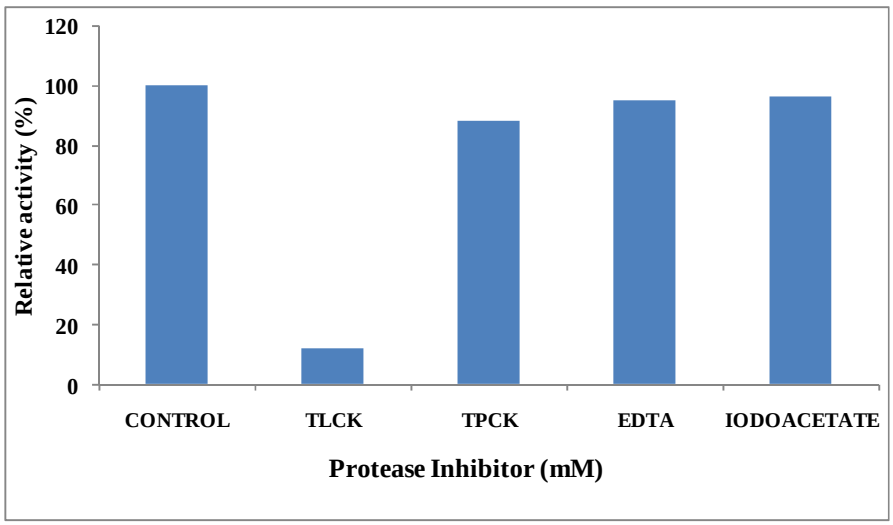
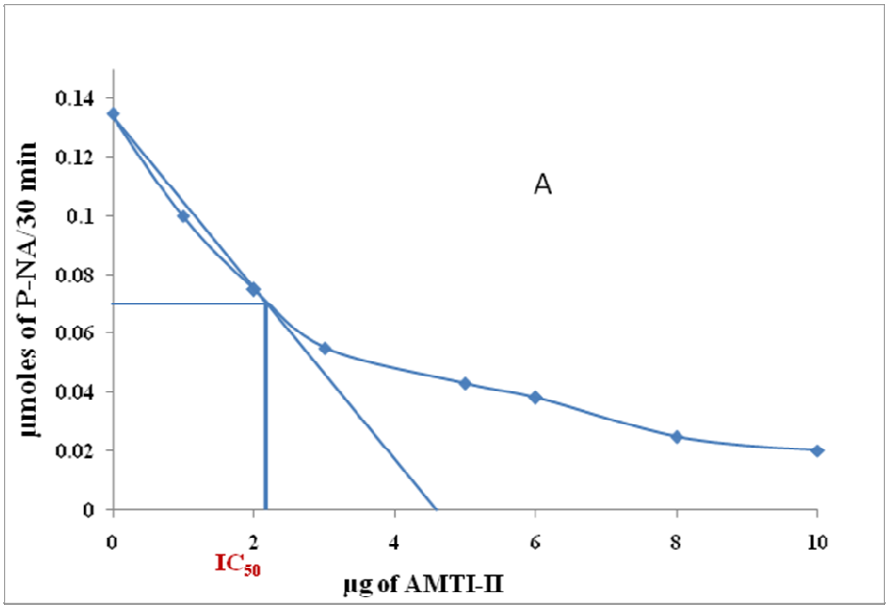


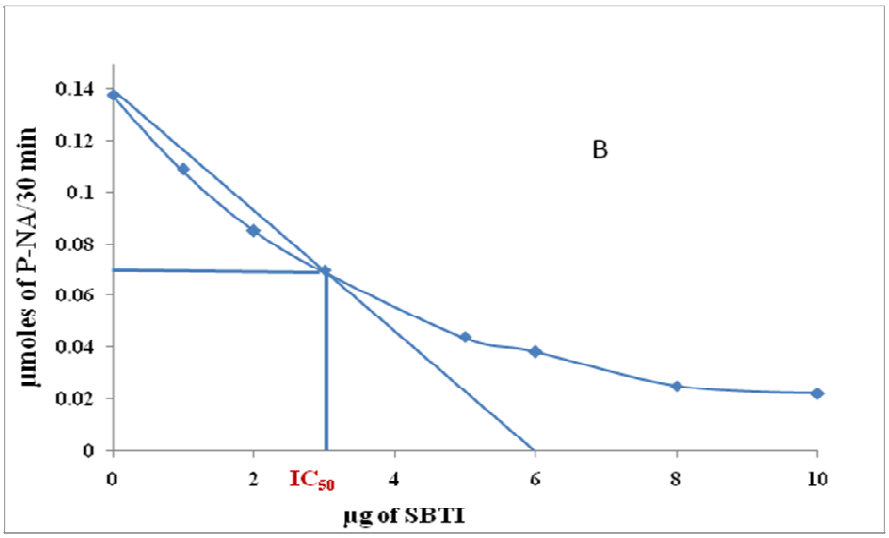
Figure 6: Effect of Protease Inhibitors on Trypsin Like Protease

Effect of AMTI-II and SBTI on Mid Gut Protease of *H. armigera*

The effect of plant proteinase inhibitors SBTI and AMTI-II, on the activity of isolated mid gut protease is shown in Figure 7. Inhibition of amidolytic activity of the enzyme was linear up to 60% -70% with the inhibitors. The amount of the inhibitor required to cause 50% inhibition of enzyme activity was 2.2 µg and 3.0 µg for AMTI-II and Soybean trypsin inhibitor (SBTI) respectively. On quantitative basis, AMTI-II was found to be most effective in inhibiting the enzyme than SBTI. AMTI-II showed strong inhibitory activity against the mid gut protease with an IC_{50} value of 2.2 µg. From the results obtained, it is clear that AMTI-II is superior to SBTI in its insecticidal effect on *H. armigera*.



(A) Inhibition of the Protease by AMTI-II



(B) Inhibition of the Protease by SBTI

Figure 7: Inhibitory Activity of Proteinase Inhibitors against *H. armigera* Protease

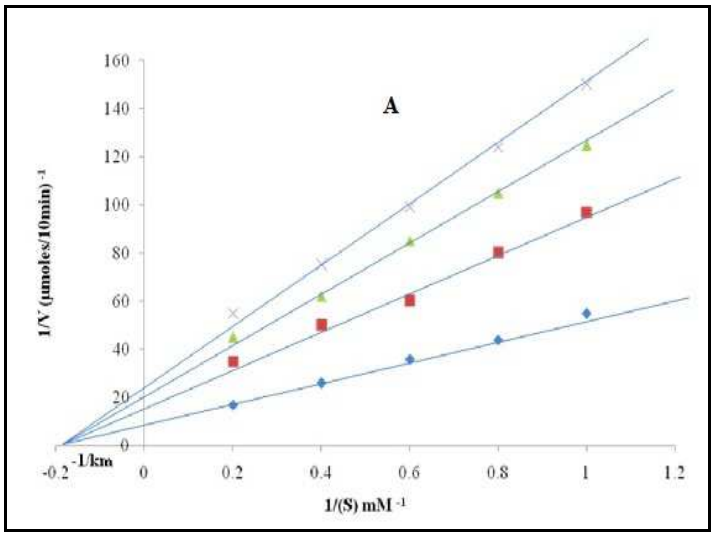
20 µg of the purified enzyme was incubated with varying amounts (1.0-10µg) of AMTI-II and SBTI for 30min at 40^{o} C. The residual enzyme activity was assayed using BAPNA as the substrate. µmoles of p-nitro aniline (µmoles of P-NA) liberated is taken as measure of enzyme activity. The amount of the inhibitor required to cause 50% inhibition of the enzyme activity was determined from the plot.

Mode of Inhibition of AMTI-II

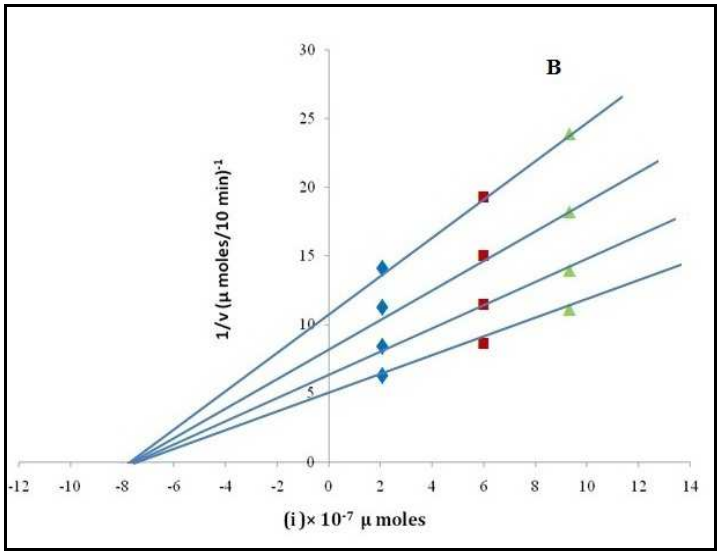
Experiments were carried out to study the effect of AMTI-II on the mid gut protease of *Helicoverpa armigera*. The protease activity in the absence and presence of AMTI-II (1 µg, 2 µg and 3 µg) was measured at different concentrations (0.9-4.0 mM) of BAPNA. The enzyme followed Michaelis-Menten kinetics. The double reciprocal plots of

the kinetics data are shown in Figure 8. In the presence of AMTI-II, a decrease in V_{max} was exhibited. K_m value and V_{max} for the protease were determined to be 3.84 x 10^{-3} M BAPNA and 45 nmol/10min/mg protein respectively. The mode of inhibition of the inhibitor was non-competitive. The K_i value of AMTI-II determined from the Dixon plots for the protease was 8 x 10^{-8} M. This low K_i value indicates that the mid gut protease has more affinity towards AMTI-II. The Ki value of SBTI (Soybean trypsin inhibitor) determined from the Dixon plots for the protease was found to be 11.2 x 10^{-8} M (Figure 9). It is clear from the inhibitory constants that the isolated mid gut protease has more affinity for AMTI-II than SBTI.

The results in the present kinetic studies suggest that the trypsin inhibitor, AMTI-II strongly inhibited mid gut protease of *Helicoverpa armigera* with a low Ki value indicating high potency of inhibitor towards the isolated mid gut protease.



(A) Lineweaver-Birk Plot



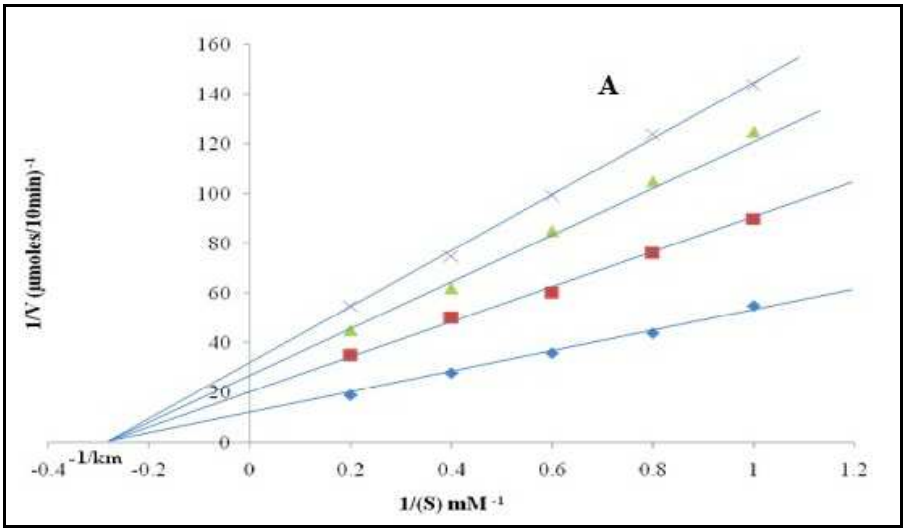
(B) Dixon Plot

Figure 8: Mode of Inhibition of *Helicoverpa Armigera* Protease by AMTI-II

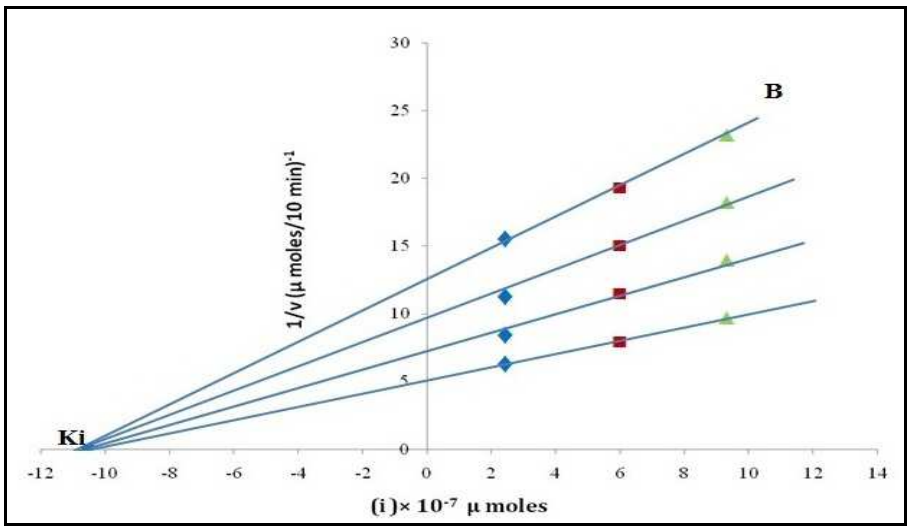
Inhibition of the amidolytic activity of the protease by AMTI-II was studied by incubating 20 µg of the purified

enzyme BAPNA (0.9-4.0mM) in a reaction system containing 1-5 µg of AMTI-II

- (-♦-) Without AMTI-II (-▲-) 2 µg of AMTI-II
- (-■-) 1 µg of AMTI-II (-×-) 3 µg of AMTI-II



(A) Lineweaver-Birk Plot



(B) Dixon Plot

Figure 9: Mode of Inhibition of *H. armigera* Protease by SBTI

Inhibition of the amidolytic activity of the protease by SBTI was studied by incubating 20 µg of the purified enzyme BAPNA (0.9-4.0mM) in a reaction system containing 1-5 µg of SBTI

- (-♦-) Without SBTI (-▲-) 2 µg of SBTI
- (-■-) 1 µg of SBTI (-×-) 3 µg of SBTI

DISCUSSIONS

The defensive capacities of plant protease inhibitors rely on inhibition of proteases present in insect guts or secreted by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development (De Leo *et al.,* 2002). Protease inhibitors have been extensively studied because of their capacity to protect plants against insect pest digestive enzymes. Digestive proteases of insects can be classified as serine, cysteine, aspartic and mettaloproteases (Terra and Ferreira, 1994) and they catalyze the release of peptides and free amino acids from dietary proteins in insect gut to meet the nutritional requirements (Amirhusina *et al.,* 2007). Srinivasan et al. (2005) carried out a study on the midgut enzymes of various pests that belong to Lepidoptera and reported that serine proteases dominate the larval guts environment and contribute to 95% of total digestive enzymes in Lepidoptera. Due to the wide distribution of these enzymes in insect plant pathogens, the serine protease inhibitors represent a mode of protection with a wide range of action (Christeller and Laing, 2005) with trypsin inhibitors being the most common type (Lawrence and Koundal, 2002).

In plants, proteinase inhibitors confer resistance against insect pests by inhibiting larval mid gut proteases. AMTI-II, the potent trypsin inhibitor, has been isolated and purified to apparent homogeneity from the seeds of *Abelmoschus moschatus* following conventional methods of protein purification and its molecular weight was found to be 21.2 kDa by SDS-PAGE. AMTI-II was found to be glycoprotein with neutral sugar content of 4% and is devoid of free sulphhydryl groups. AMTI-II was quite stable up to 80 °C for 10 min and was not affected at alkaline as well as acidic conditions tested. Treating with 8 M urea and 1% SDS for 24 h at room temperature did not result in any loss of its anti-tryptic activity (Muni Kumar *et al.,* 2015).

AMTI-II is unique in that it exhibited hem agglutinating activity towards different human and animal erythrocytes. The lectin activity of AMTI-II was found to be inhibited by D-galactose only. The lectin activity was stable over a broad pH range (3.0-12.0) and temperature up to 60^{o}C for 15 min and was also resistant to denaturants. Oxidation of AMTI-II by sodium metaperiodate and treatment with PNGase F affected its lectin activity (Muni Kumar *et al.,* 2014). Proteinase inhibitor and lectin genes have been reported as attractive tools for increasing insect/pest resistance among crop plants (Amirhusin *et al.,* 2004; Abdeen *et al.,* 2005).

A combination of proteinase inhibitory and lectin activities residing in a single protein could be harmful nutritionally, but is more useful in raising the defense potential in plants. Hence, in the present study, the multifunctional trypsin inhibitor, AMTI-II with antitryptic, lectin and antimicrobial properties has been checked for its efficacy against isolated mid gut protease of *Helicoverpa armigera*.

Trypsin- like protease has been purified from the mid guts of *Helicoverpa armigera* using ion exchange chromatography and gel filtration on QAE- sephadex column. The protease activity was found to reside in two protein peaks. High BAPNAase activity was associated with a second peak eluted with 0.3M NaCl. The enzyme was found to be homogeneous by the criteria of native PAGE. The specific activity of the purified enzyme was 1426.92 TU per mg protein. The final yield was 52.81% with a fold purification of 3.81. SDS-PAGE analysis in the presence of 2-mercaptoethaonol gave a single band corresponding to a molecular weight of about 30.4 kDa. The protein peak obtained in the wash through fractions could thus be a polymerized form of the protease itself or association of the protease with other mid gut proteases.

Proteases with molecular weights ranging from 41.6 - 172.9 kDa from mid gut extracts of the insect were reported by Johnston et al. (1991) and Harsulkar et al. (1998). Telang et al. (2005) reported two trypsin like proteases in the mid

guts of *Helicoverpa armigera* with molecular weights 24 and 29kDa. A trypsin like protease from *Helicoverpa armigera* gut was purified 37 fold with 22% yield and its molecular weight was found to be 18.8 kDa (Grover *et al.,* 2016).

The isolated protease from *Helicoverpa armigera* was found to be susceptible to inhibition by the two plant proteinase inhibitors tested. AMTI-II strongly inhibited the protease. The IC_{50} values for AMTI-II and Soybean trypsin inhibitor (SBTI) were found to be 2.2 µg and 3.0 µg respectively. On quantitative basis, AMTI-II was found to be most effective in inhibiting the enzyme than SBTI. AMTI-II showed strong inhibitory activity against the mid gut protease with a low IC_{50} value than SBTI. From the results obtained, it is clear that AMTI-II is superior to SBTI in its insecticidal effect on *Helicoverpa armigera.*

The mode of action of AMTI-II with the mid gut protease was found to be non competitive. Results of kinetic studies revealed a low Ki value, 8×10^{-8} M. for AMTI-II. With SBTI, Ki value obtained was 11.2×10^{-8} M. Investigations of Johnston *et al.*, (1991) demonstrated that SBTI, BBI (Bowman-Birk serine proteinase inhibitor) inhibited this protease non-competitively. On the contrary, chickpea trypsin inhibitor (CPTI) and lima bean trypsin inhibitor (LBTI) inhibited the protease competitively. Trypsin inhibitor from *Achyranthes aspera* (AATI) inhibited the protease non-competitively with a low Ki value of 8.2×10^{-8} M (Geeta, 2011). JSTI also inhibited the enzyme non-competitively with a Ki value of 7.8×10^{-8} M (Sudha, 1999). CPTI and CPTCI inhibited the *Helicoverpa armigera* protease in a competitive manner with Ki values 6.9×10^{-8} M and 2.9×10^{-7} M respectively (Godbole *et al.,* 1994).

Several *in vitro* studies related to determining the efficacy of various proteinase inhibitors against mid gut proteinases of *Helicoverpa armigera* have been reported in addition to feeding experiments. Kansal et al. (2008a) reported that 95% inhibition of larval gut proteinase of *Helicoverpa armigera* by the trypsin inhibitor present in seed extract of soybean. Soybean Kunitz type trypsin inhibitor (SBTI) and Soybean Bowman–Birk type trypsin-chymotrypsin inhibitor (SBBI) have been shown to reduce larval weights of *Helicoverpa armigera* in artificial diet (Johnston *et al.,* 1993). Soybean trypsin inhibitor also affects the growth and digestive physiology of *Helicoverpa armigera* (Wang *et al.,* 1996).

Kumar et al. (2007) reported protease inhibitor proteins in seed extracts of various legumes specific against *Helicoverpa armigera* gut proteinase (HGP). Patankar et al. (1999) and Srinivasan et al. (2005) reported an inhibition of *Helicoverpa armigera* gut proteinase activity by proteinase inhibitors from the seeds of chickpea by 2–33 and 60%, respectively. Harsulkar et al. (1999) showed an *in vitro* inhibition of *Helicoverpa armigera* gut proteinase activity by 0–55% in host plants, whereas total inhibition of *Helicoverpa armigera* gut proteinase activity in non-host plants. It is also evident from the studies that the extent of inhibition of *Helicoverpa armigera* gut proteinase by the kidney bean trypsin inhibitor was greater than several other reports. Nair et al. (2013) reported insecticidal potential of trypsin inhibitor from *Cicer arietinum* seeds against *Helicoverpa armigera* by conducting both *in vitro* and feeding experiments.

The anti nutritional effect of protease inhibitor depends on its affinity for insect mid gut protease and its susceptibility to cleavage by non target enzymes in the gut canal. When incubated with mid gut extracts, AMTI-II was found to be stable and resistant to degradation by digestive protease of larval *Helicoverpa armigera* as tested by its electrophoretic pattern. Thus, the effect of AMTI-II on mid gut proteases alone is to be considered for its insecticidal property and the presence of it in tissues can provide protection to plants against this notorious insect pest.

CONCLUSIONS

The results obtained in the present investigation support AMTI-II as a potential insecticidal agent as powerful as

SBTI against mid gut protease of *Helicoverpa armigera.* Since trypsin inhibitors and lectins have been shown to provide protection in plants against invading insect pests and pathogens, AMTI-II possessing antitryptic, lectin and insecticidal activity against mid gut protease of *Helicoverpa armigera* can be explored in the agricultural front for developing transgenic. Hence, the protease inhibitor, AMTI-II could be considered as potential candidate for its use in genetic transformation of crops for pest management after carrying out extensive *in vivo* studies against mid gut proteases of insect pests including *Helicoverpa armigera.*

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